VIROLOGY 213, 558–568 (1995)

Molecular Basis of Antigenic Variation between the Glycoproteins C of Respiratory Bovine Herpesvirus 1 (BHV-1) and Neurovirulent BHV-5

S. I. CHOWDHURY

Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506

Received July 20, 1995; accepted September 11, 1995

Herpesvirus glycoprotein C (gC) functions as a major virus attachment protein. The gC sequence of the neurovirulent bovine herpesvirus type 5 (BHV-5) virus was determined and compared with the gC sequence of the nonneurovirulent BHV-1. Alignment of the predicted amino acid sequences of BHV-1 and BHV-5 gC ORFs showed that the amino-terminal third of the protein differed between the two viruses. Whole or subgenomic fragments of gC coding regions from both viruses were expressed as trpE–gC fusion proteins in *Escherichia coli* to map linear epitopes defined by type-specific murine monoclonal antibodies (MAbs). Based on the reactivity of BHV-1-specific MAbs with the recombinant proteins, two epitopes were mapped between BHV-1 gC residues 22 and 172. Unidirectional deletion of these residues at the carboxy end mapped one within residues 22–69 and the other within residues 103–122. Two BHV-5-specific MAbs identified an epitope coding region within BHV-5 gC residues 31–78. Bovine antisera against BHV-1 and BHV-5 showed specificity to BHV-1 gC residues 22–69 and to BHV-5 gC residues 31–78, respectively, in a type-specific manner.

INTRODUCTION

Bovine herpesvirus-1 (BHV-1) is associated with a variety of clinical diseases including rhinotracheitis, conjunctivitis, genital infections, and occasionally abortion, enteritis, encephalitis, and generalized systemic infections in cattle (Ludwig, 1983; Wyler *et al.,* 1989). BHV-1 strains fall into two different groups based on their DNA restriction profiles and pathogenic properties: the respiratory/abortogenic group (infectious bovine rhinotracheitis virus) and the nonabortogenic genital group (infectious pustular vulvovaginitis). The neurovirulent bovine herpesvirus (previously classified as BHV-1.3) has been reclassified recently as BHV-5 (Roizman *et al.,* 1992) and is known to cause epidemics of a uniformly fatal encephalitis in calves (Carillo *et al.,* 1983a,b; French, 1962; Eugster *et al.,* 1974; Bartha *et al.,* 1969). Both the BHV-1 and the BHV-5 strains are neurotropic viruses, but they differ in their ability to cause encephalitis in calves (Belknap *et al.,* 1994). BHV-1 grows in higher titers in the respiratory epithelium compared to BHV-5 (Belknap *et al.,* 1994), but a glycoprotein C (gC)-deleted BHV-1 vaccine strain replicates less efficiently in the upper respiratory tract of cattle (Liang *et al.,* 1992).

Herpesvirus glycoproteins are important for the interaction of the virus with its host. The glycoproteins mediate infection of target cells and, thus, influence cell and tissue tropism and are also major antigens recognized by the infected host's immune system (Babiuk *et al.,* 1987; Little *et al.,* 1981; Glorioso *et al.,* 1984; Herold *et al.,* 1991; Lupton and Reed, 1980). The alpha herpesviruses, including herpes simplex viruses (HSV-1, HSV-2), pseudorabies virus, and BHV-1, contain a number of functionally and structurally homologous glycoproteins. Three major glycoproteins of BHV-1 (gB, gC, and gD, previously named gI, gIII, and gIV, respectively) are partially homologous to HSV gB, gC, and gD, respectively.

Bovine herpesvirus types 1 and 5 share 85% DNA homology when the whole genome is compared (Engles *et al.,* 1987). However, the DNA restriction maps of BHV-1 and BHV-5 are strikingly distinct (Engles *et al.,* 1987; Bulach and Studdert, 1990). This suggests that nucleotide sequence differences between these two viruses are distributed throughout the entire genome. Based on monoclonal antibody (MAb) reactivities, we and others have demonstrated antigenic differences between the major glycoproteins of these two viruses (Metzler *et al.,* 1985, 1986; Friedli and Metzler, 1987; Collins *et al.,* 1993; Abdelmagid *et al.,* 1995). However, gC of these two viruses was the most dissimilar (Collins *et al.,* 1993).

BHV-1 gC is a 180/97K dimeric glycoprotein. The protein is important in the virus attachment process because purified gC inhibits BHV-1 attachment (Collins *et al.,* 1984; Marshall *et al.,* 1988; van Drunen Little-van den Hurk *et al.,* 1984; van Drunen Little-van den Hurk and Babiuk, 1986). BHV-1 gC consists of 521 amino acid (aa) residues that constitute a signal peptide between residues 7 and 21, a projecting region, and the transmembrane anchor domain between residues 467 and 500 followed by a hydrophilic C-terminal region (Fitzpatrick *et al.,* 1989). Four potential N-linked glycosylation sites [Asn-X-Ser (Thr)] are present on the predicted amino acid

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sequences of gC located at amino acids 93, 111, 164, and 208 (Fitzpatrick *et al.,* 1989). Based on competitive binding assays using gC-specific MAbs, one unique and four interrelated antigenic areas of BHV-1 gC were reported (Marshall *et al.,* 1988). Recently, three BHV-1 gC epitopes have been mapped between aa residues 22 and 150 (Fitzpatrick *et al.,* 1990). However, the precise location of each of these three epitopes was not determined. Two other epitopes were mapped between residues 140–240 and 230–287 (Fitzpatrick *et al.,* 1990).

The objectives of this study were to (i) analyze the genetic basis of antigenic differences between the gC genes of nonneurovirulent/respiratory BHV-1 and neurovirulent BHV-5 viruses and (ii) identify and analyze several BHV-1 gC- and BHV-5 gC-specific epitopes located along the gC coding region. The BHV-5 gC sequence was determined and compared with the BHV-1 gC sequence. DNA fragments containing the whole and partial sequences of BHV-1 and BHV-5 gC coding regions were expressed as trpE–gC fusion proteins in *Escherichia coli.* BHV-1 and BHV-5 gC-specific MAbs were reacted to respective fusion proteins by Western immunoblotting, and the precise locations of two BHV-1-specific and one BHV-5-specific linear epitopes were determined by analyzing MAb reactivity.

MATERIALS AND METHODS

Virus strains and cell lines

The Cooper (Colorado-1) strain of BHV-1 was obtained from the American Type Culture Collection (Rockville, MD). The TX-89 strain of BHV-5 (isolated from a case of viral encephalitis) (d'Offay *et al.,* 1993) was provided by Dr. d'Offay from Oklahoma State University. The two viruses were propagated and titrated in Madin–Darby bovine kidney cells grown in Dulbecco modified Eagle's medium supplemented with 10% fetal bovine serum.

Monoclonal and polyclonal antibodies

MAbs F2 and 24 are specific for BHV-1 gC (Abdelmagid *et al.,* 1993; Collins *et al.,* 1993). They neutralized BHV-1 but not BHV-5 in the presence of complement (Collins *et al.,* 1993; S. I. Chowdhury, unpublished data). MAbs 8B1 and L6G are BHV-5 gC specific but are nonneutralizing (Abdelmagid *et al.,* 1993; Chung *et al.,* 1994). MAbs 24 and 8B1 were provided by Dr. H. Minocha of Kansas State University. MAbs F2 and L6G were obtained from Dr. J. Collins, Colorado State University. Monospecific bovine anti-BHV-1 and anti-BHV-5 sera (Collins *et al.,* 1993) were obtained from Dr. Whetstone, National Animal Disease Center, USDA.

Mapping, cloning, and sequencing of the BHV-5 gC gene

The location of the gC gene on the BHV-5 genome and pertinent restriction sites for the subcloning and the sequencing strategy are illustrated in Fig. 1. A pUC-based plasmid library containing the *Bam*HI genomic fragments of the BHV-5 genomic DNA was developed. To verify the general location of the BHV-5 gC gene, the 1.7-kb *Bam*HI–*Afl*III subfragment, carrying the gC gene sequences of BHV-1, was subcloned from the *Hin*dIII I fragment of BHV-1 DNA (Mayfield *et al.,* 1983). The above cloned DNA was labeled with ^{32}P by nick translation (Sambrook *et al.,* 1989) and used as a probe for hybridization (Chowdhury *et al.,* 1988) with the 18.95-kb *Bam*HI A fragment of BHV-5 (Engles *et al.,* 1987; Bulach and Studdert, 1990). To precisely map the BHV-5 gC gene, the *Bam*HI A fragment was digested further with several restriction endonucleases, separated on agarose gels, and analyzed by hybridization with the same probe. Based on the hybridization results, a 1.72-kb *Bam*HI–*Afl*III fragment (m.u. 0.135–0.125) was identified and cloned. A restriction map of the cloned 1.72-kb fragment was constructed, and subclones spanning the entire fragment were generated. Both strands of each fragment were sequenced by Sanger's dideoxy chain termination method (Sanger *et al.,* 1977) and verified by the method of Maxam and Gilbert (1977; Chowdhury *et al.,* 1990).

Sequence analysis

DNA sequence data were assembled and analyzed using the Seqaid II sequence analysis software (D. D. Rhodes and D. J. Roufa, Center for Basic Cancer Research, Kansas State University, Manhattan). The predicted amino acid sequences of BHV-5 gC (this study) and BHV-1 gC (Fitzpatrick *et al.,* 1989) were aligned by using Multalin software (Corpet, 1988). Hydropathicity analyses were performed (Kyte and Doolittle, 1982) using a nine-aa window, and the antigenicity profiles of predicted aa sequences were analyzed using the Seqaid II software.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis

SDS–PAGE of mock- and virus-infected cell proteins or bacterially expressed fusion proteins was performed under reducing conditions as described earlier (Laemmli, 1970; Abdelmagid *et al.,* 1995).

Construction of recombinant plasmids

The 1.7-kb *Bam*HI/*Afl*III (blunt) fragment containing the whole BHV-1 gC ORF (Fitzpatrick *et al.,* 1989) was cloned into the *Bam*HI/*Sma*I site of a pATH 23 plasmid (Koerner *et al.,* 1991) to yield an inframe trpE–BHV-1 gC fusion gene (plasmid clone p1gC1-521). The recombinant plasmid was used to transform *E. coli* RR1 cells. A clone containing the BHV-1 gC coding sequences (verified by sequencing) was selected and tested for gC expression

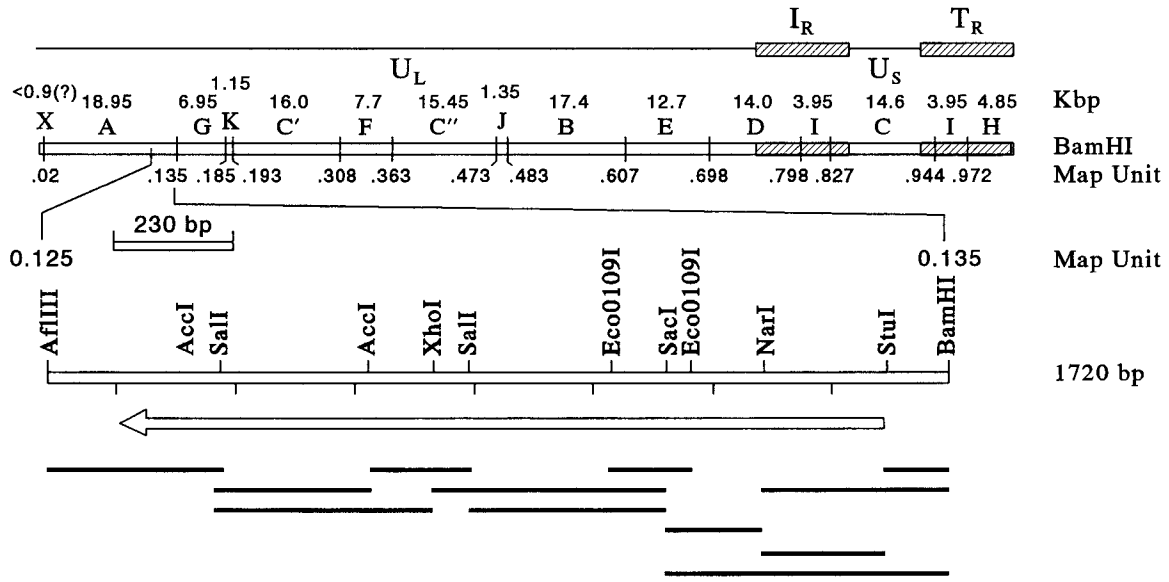

FIG. 1. The *Bam*HI restriction site map of BHV-5 DNA and the cloning and sequencing strategies of BHV-5 gC gene. The genomic organization of BHV-5 (TX-89 strain), depicted on top, consists of unique long (U_L) and short (U_S) regions and two repeat regions (I_R and T_R). The *Bam*HI restriction endonuclease map was described originally for the N569 strain of BHV-5 by Engles *et al.*, 1987. The gC gene of BHV-5 was mapped to the *Bam*HI A (18.95 kb) genomic fragment between m.u. 0.02 and 0.135 and then to the *Bam*HI/*Afl*III fragment (1720 bp) between m.u. 0.125 and 0.135 by subcloning and hybridization with radiolabeled BHV-1 gC gene DNA probe. A restriction site map of the *Bam*HI/*Afl*III (1720 bp) fragment was generated, and various restriction endonuclease subfragments, indicated as bold lines, were cloned and sequenced. The arrow represents the BHV-5 gC ORF, which is 1415 nucleotides long starting with the ATG codon and terminating with TAG. The arrow shows the direction of transcription.

(described below). All subsequent clonings and manipulations spanning portions of the BHV-1 gC open reading frame (ORF) were generated either directly or indirectly from this clone. The 1.01-kb *Bam*HI/*Xho*I fragment containing the first 300 aa of BHV-5 gC (Fig. 2) was inserted into the *Bam*HI/*Sal*I site of a pATH 22 plasmid (Koerner *et al.*, 1991) to yield an inframe trpE–BHV-5 gC fusion gene and cloned by transforming *E. coli* RR1 cells. A clone (plasmid clone p5gC1-300) containing the BHV-5 gC coding sequences (verified by sequencing) was selected, and the specificity of the induced fusion protein to a BHV-5 gC-specific MAb (MAb 8B1) was tested (see Results). All subsequent clonings containing portions of the BHV-5 gC ORF were generated either directly or indirectly from this clone.

Construction of p1gC1-336. Plasmid p1gC1-521 was digested with *Xho*I and treated with Klenow enzyme. The DNA was redigested with *Bam*HI, and the 1.16-kb fragment was gel purified and cloned into *Bam*HI/*Sma*I-digested pATH23 plasmid.

Construction of p1gC1-172. Plasmid p1gC1-336 was digested with *Sst*I, and the larger fragment was purified and religated. The resulting plasmid contain the *Bam*HI/*Sst*I fragment coding the N-terminal 172 aa of BHV-1 gC.

Construction of p1gC173-336. Plasmid p1gC1-336 was digested with *Sst*I, and the 500-bp fragment was purified and cloned into the *Sst*I site of the pATH22 plasmid. A clone containing the BHV-1 gC residues 173–336 in correct orientation (verified by sequencing) was selected.

Construction of p1gC1-122, p1gC1-103, and p1gC1-69. These plasmids were obtained by unidirectional deletion at the 3′ end of gC coding sequences contained within the plasmid clone p1gC1-172. To facilitate unidirectional deletion, the *Xba*I/*Eco*RI fragment of p1gC1-172 flanking the *Bam*HI/*Sst*I sites of insert was first cloned into the *Xba*I/*Eco*RI sites of pGEM 7Z (clone p7ZgC1-172). Then unidirectional deletions were introduced at the *Eco*RI site following the protocol for exonuclease III at 30° (Erase-a-Base system; Promega). Samples were taken at 1-min intervals and processed further following the manufacturer's instruction. Following ligation, several deleted plasmids were used to transform *E. coli* DH5α cells. Clones containing deletions were screened, and several clones were sequenced to determine the extent of the deletions. Based on the sequences, three clones (p1.1, p2.2, and p3.7 with 151-, 208-, and 310-bp deletions with respect to the *Sst*I site of p7ZgC1-172) were selected for further cloning into the pATH23 vector. Plasmids p1.1, p2.2, and p3.7 were digested with *Xba*I/*Sst*I. The 530-, 470-, and 370-bp fragments (p1.1, p2.2, and p3.7, respectively) were cloned into *Xba*I/*Sst*I-digested pATH23 plasmid to yield p1gC1-122, p1gC1-103, and p1gC1-69, respectively.

Construction of p5gC1-136. Plasmid p5gC1-300 was digested with *Sst*I, and a 520-bp fragment (*Sst*I site of pATH 22 is located immediately upstream of the *Bam*HI site) was purified and inserted into the *Sst*I-digested pATH 22 plasmid (in correct orientation).

Construction of p5gC1-78. Plasmid p5gC1-136 was di-

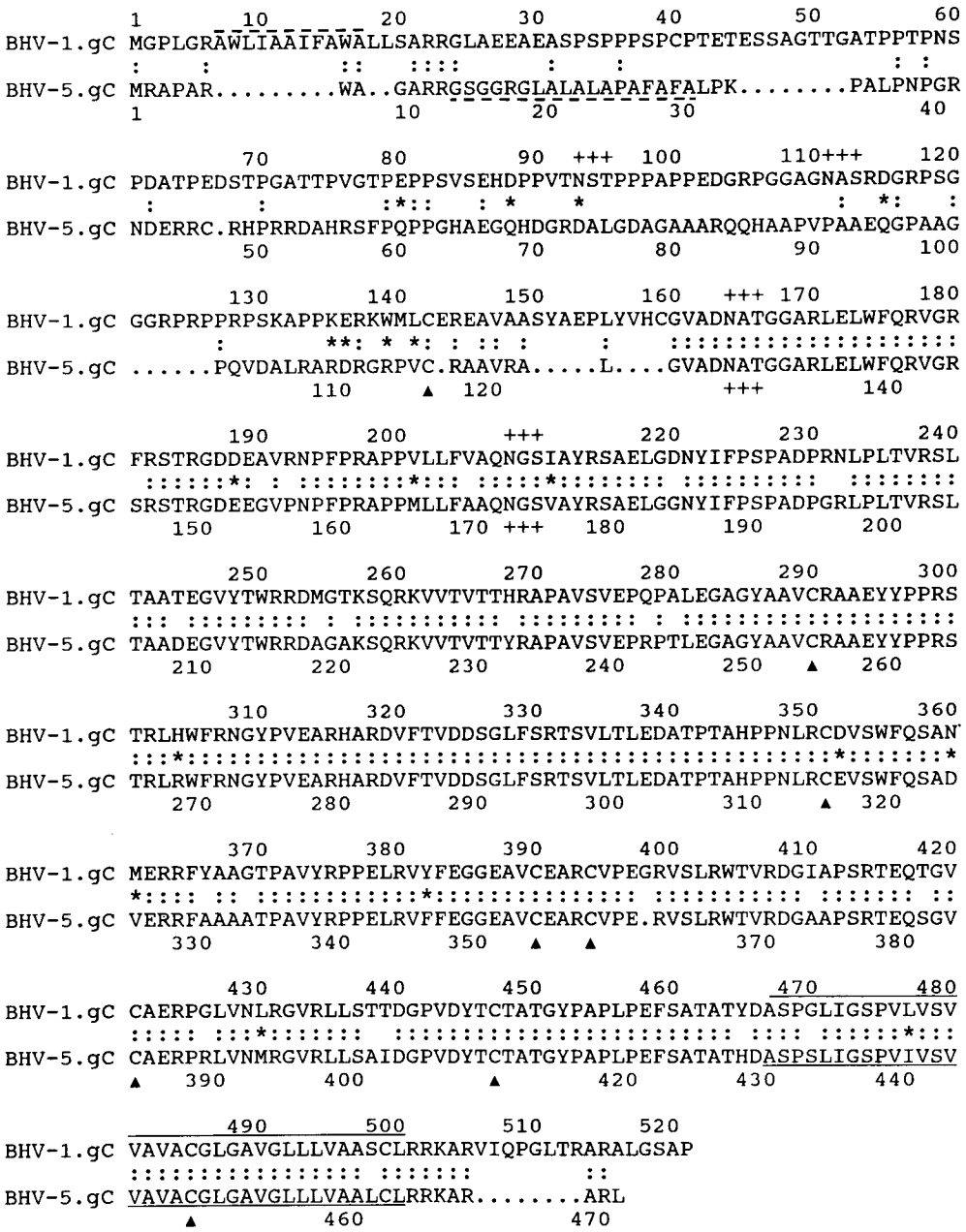

FIG. 2. Comparison of the predicted amino acid sequence of the BHV-5 gC with the predicted amino acid sequences of BHV-1 gC (Fitzpatrick *et al.*, 1989). The predicted amino acid sequences of the BHV-1 and BHV-5 glycoproteins C were aligned by using Multalin software (Corpet, 1988). Amino acid numbering starts at the first methionine and is indicated for BHV-1 (top) and BHV-5 (bottom). Amino acids aligned identically between the two sequences are indicated by two dots, conservative substitutions are indicated by an asterisk, and conserved cysteine residues are marked by an arrowhead. Potential N-linked glycosylation sites are indicated (+++). The presumptive signal sequences and the transmembrane anchor sequences are shown by broken and solid lines, respectively.

gested with *Nar*I and treated with the Klenow enzyme. The DNA was redigested with *Sst*I, and a 350-bp fragment was purified and inserted into the *Sst*I/*Sma*I-digested pATH22 plasmid.

Preparation of fusion proteins

The synthesis of trpE–gC fusion proteins was induced in modified M9 medium by tryptophan starvation and indoleacrylic acid as described (Koerner *et al.*, 1991; Abdelmagid *et al.*, 1995).

RESULTS

Identification of BHV-5 gC open reading frame and comparison of predicted aa sequences of BHV-5 and BHV-1 gC

The nucleotide (nt) sequence of the BHV-5 gC gene has been deposited with the GenBank Database under

Accession No. U35883. The nucleotide sequence analysis of the 1.72-kb *Bam*HI/*Afl*III fragment (m.u. 0.135–0.125) identified two potential start codon (ATG) sequences situated close to each other at positions nt 69 and nt 113. Translation initiation at nt 113 would result in a 1415-bp ORF coding for 470 aa residues (ORF1; Fig. 2), large enough to encode the BHV-5 gC gene but 50 aa shorter than the BHV-1 gC gene. However, translation initiation at nt 69 would result in a much shorter 641-bp ORF coding for only 213 aa (ORF2; data not shown). Alignment of BHV-5 gC ORF1 sequences with the corresponding sequences of BHV-1 (Fitzpatrick *et al.*, 1989) showed considerable homology (89.9%) at the carboxy-terminal two-thirds of the two proteins (aa 124–470 of BHV-5 gC); however, the amino-terminal third of the two proteins (aa 1–123 and aa 1–149 of BHV-5 and BHV-1 gC, respectively) differed significantly (23.5% homology) (Fig. 2). Alignment of ORF2 sequences showed some homology of the first 40 aa but no obvious similarity to the rest of BHV-1 sequences (data not shown). To identify the BHV-5 gC as the product of the ORF1 sequences, the 1.01-kb *Bam*HI/*Xho*I fragment containing the first 300 aa was expressed as a trpE fusion protein in pATH22 vector, and its reactivity to a BHV-5 gC-specific MAb, 8B1, was tested by immunoblotting experiments. The MAb reacted specifically to a 70K protein corresponding to the molecular weight of the fusion product (trpE, 37K; *Bam*HI/*Xho*I, 32.26) (Fig. 3b).

As in BHV-1, the predicted BHV-5 protein has 9 cysteine residues, 8 of which are located in the putative external domain. The cysteine residue at aa 40 of BHV-1 gC did not align with cysteine residue at aa 47 of BHV-5 gC. However, 8 cysteine residues (aa 116, 254, 315, 354, 358, 384, and 448 of BHV-5 gC), including the 6 cysteine residues conserved in several α herpesviruses (Fitzpatrick *et al.*, 1989), aligned with the two sequences. Two potential N-linked glycosylation sites (Kornfeld and Kornfeld, 1985) located at aa 164 and 208 of BHV-1 gC are conserved, but two other potential glycosylation sites at aa 93 and 111 of BHV-1 gC are not present in the BHV-5 gC (Fig. 2). Hydropathic analysis of the predicted protein revealed the presence of two prominent hydrophobic peaks similar to the BHV-1 gC, representing the signal sequence and the transmembrane anchor sequence (Fig. 4). Based on empirical rules for predicting signal sequences (von Heinje, 1986), aa residues 14 to 30 have the position, length, relative hydrophobicity, and consensus cleavage site characteristic of a signal sequence. Similarly, aa residues 430 to 463 have the position, length, and relative hydrophobicity characteristics of a transmembrane anchor sequence (Figs. 2 and 4). The analysis also revealed that the relative hydrophilicity characteristics of aa 1–120 and aa 1–150 of BHV-5 gC and BHV-1 gC, respectively, showed some degree of divergence. However, with the exception of a shorter carboxy-terminal hydrophilic region in the BHV-5 sequence, located immediately downstream of the putative transmembrane domain, the rest of the sequences showed almost identical hydrophilicity profiles when aligned with the BHV-1 (Fig. 4).

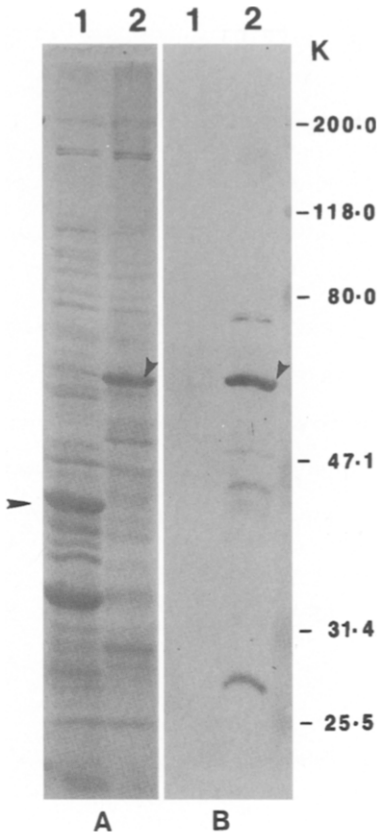

FIG. 3. Identification of BHV-5 gC ORF-specified protein. (A) Coomassie blue-stained SDS–PAGE gel containing (lane 1) trpE and (lane 2) trpE–gC (BHV-5) aa 1–300 proteins and (B) immunoblotting of the same proteins with BHV-5 gC-specific MAb 8B1. The locations of (lane 1) trpE and (lane 2) trpE–gC(BHV-5) fusion proteins are indicated by arrowheads.

Further analysis of the amino-terminal sequence between the two viruses show that there are more negatively charged residues within the first 149 aa of BHV-1, whereas the corresponding region of BHV-5 gC (aa 1–123) contained more positively charged aa. This was also true when the BHV-1 and BHV-5 sequences were analyzed without the signal sequences. Respectively, BHV-1 residues 22–149 had 12 glutamic acid, 5 aspartic acid, 10 arginine, and 3 lysine residues while the corresponding BHV-5 residues (31–123) had 3 glutamic acids, 7 aspartic acids, 15 arginines, and 1 lysine (Fig. 2).

Use of recombinant fusion proteins and virus-specific monoclonal antibodies to define epitope structure on the BHV-1 and BHV-5 gC glycoproteins

The 37-kDa trpE protein (vector control) and the larger-sized trpE–gC fusion proteins, which were composed of

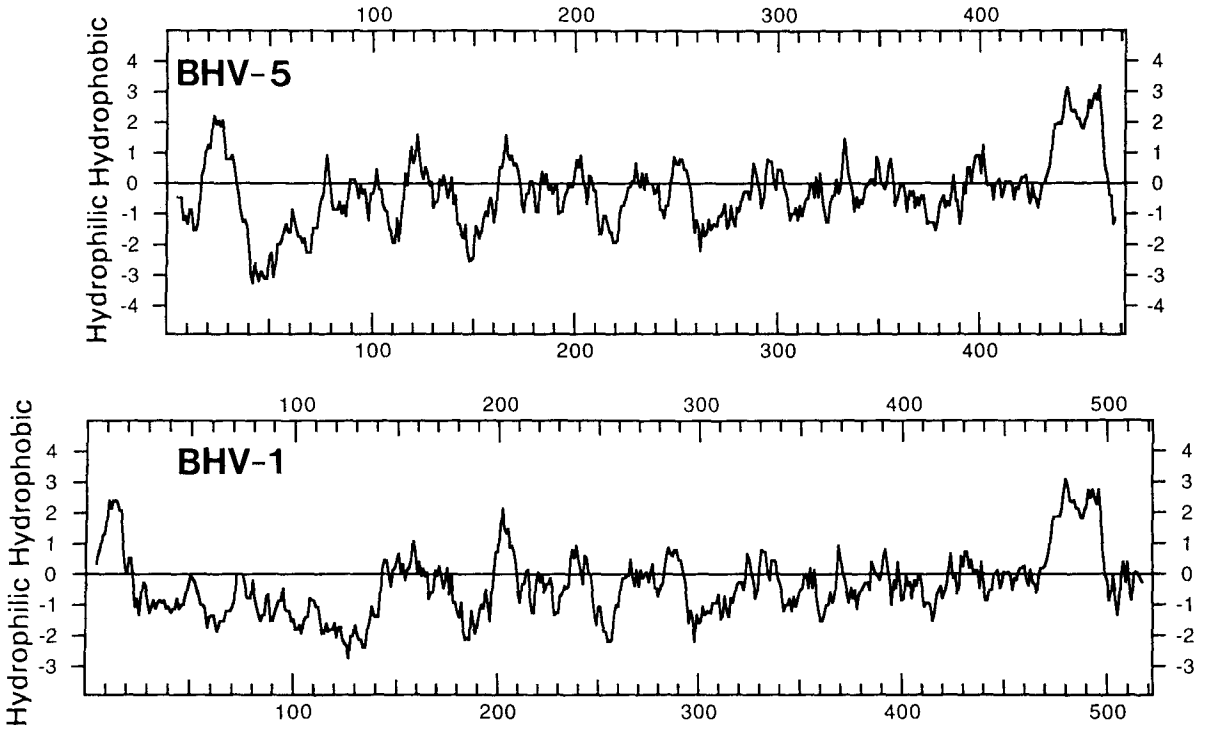

FIG. 4. Comparison of the hydropathicity plots of the amino acid sequences of BHV-1 and BHV-5 glycoproteins C. The BHV-5 and BHV-1 gC amino acid sequences were analyzed for hydropathicity characteristics (Kyte and Doolittle, 1982) using a nine-amino-acid window. The BHV-5 gC is shown on top, and the BHV-1 gC (Fitzpatrick *et al.*, 1989) is shown on the bottom. The hydropathic scores and the amino acid numbers are shown on the vertical and horizontal axes, respectively.

the trpE gene plus the gC coding regions of BHV-1 and BHV-5 of variable length, are shown in Figs. 5 and 7, respectively. The induced fusion proteins containing BHV-1 gC residues, aa 1–521 (data not shown), aa 1–336 (data not shown), aa 1–172, aa 1–122, aa 1–103, and aa 1–69 were represented by p1gC1-521, p1gC1-

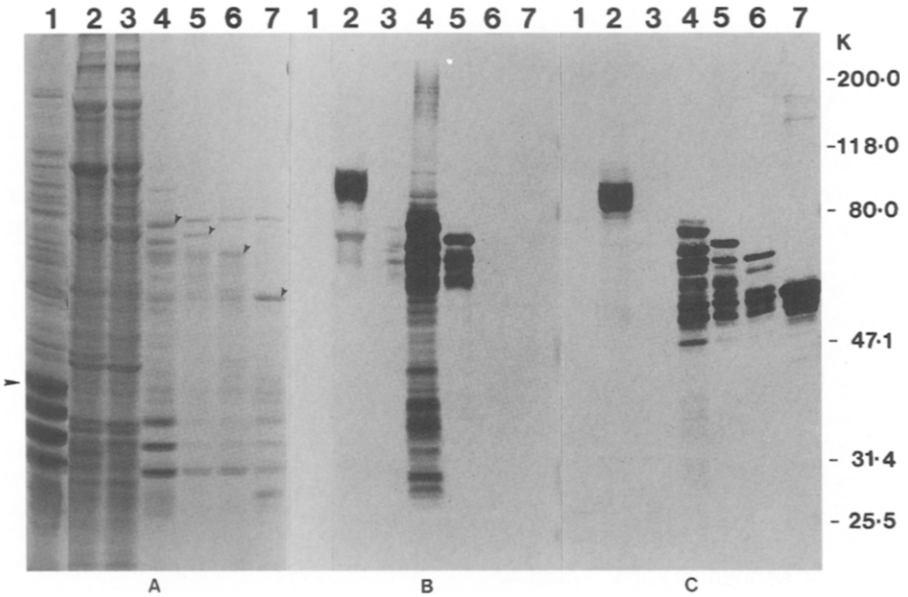

FIG. 5. SDS–PAGE and immunoblotting analysis of trpE–gC (BHV-1) fusion proteins. (A) Coomassie blue-stained gel showing fusion proteins expressed by subfragments of gC (BHV-1) ORF using the pATH vector system. (Lane 1) trpE, (lane 2), BHV-1, and (lane 3) BHV-5 infected MDBK cell lysates; trpE–gC (BHV-1) proteins, (lane 4) 1–172, (lane 5) 1–122, (lane 6) 1–103, and (lane 7) 1–69. The locations of trpE (lane 1) and trpE–gC (BHV-1) fusion proteins (lanes 4–7) are marked with arrowheads. Immunoreactivity of the same proteins with (B) MAb 24 and (C) MAb F2.

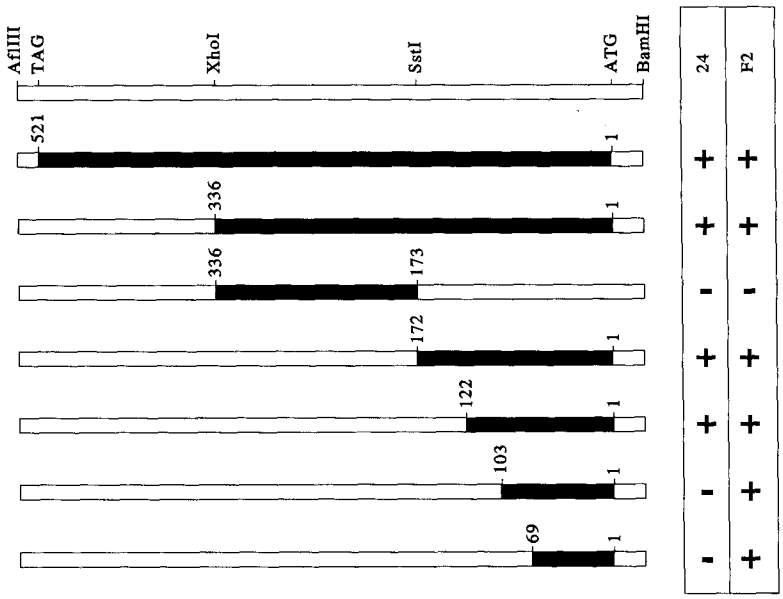

FIG. 6. Schematic representation of the BHV-1 gC glycoprotein and its segments that were expressed in pATH vectors. The *Bam*HI/*Afl*III fragment containing the gC (BHV-1) ORF is depicted on top, showing restriction endonuclease sites used for cloning into pATH plasmids. Glycoprotein C protein segments expressed are shown as solid bars identified by aa numbers. A summary of the immunoreactivity of gC (BHV-1) protein segments with MAbs 24 and F2 is shown on the table to the right, with (+) to denote reactivity and (−) for no reactivity.

336, p1gC1-172, p1gC1-122, p1gC1-103, and p1gC1-69, respectively (Fig. 5A). Fragments containing the epitope-bearing sequences in the correct reading frames were recognized by the MAbs, thus localizing the epitope region in the glycoprotein. The results of immunoblotting of these fusion proteins with two BHV-1 gC-specific MAbs are shown in Figs. 5B and 5C. In addition to the fusion proteins with predicted molecular weight, a number of additional bands also were detected consistently by Western blotting. One explanation for the presence of these bands is proteolytic degradation of exogenous proteins, which has been reported for *E. coli* (McDonald *et al.*, 1987).

The reactivity of the fusion proteins with the MAbs and the location of the epitope-bearing segment on the BHV-1 gC ORF are summarized in Fig. 6. MAbs F2 and 24 reacted to the fusion proteins expressing the aa residues 1–521 (entire gC ORF; data not shown), 1–336 (data not shown), and 1–172 (N-terminal third) (Fig. 5) but not to the fusion protein expressing residues 173–336 (data not shown). This narrowed the location of epitopes to a stretch of 172 aa expressed by the *Bam*HI/*Sac*I subfragment (Figs. 5 and 6). Unidirectional deletions of residues 1–172 at the carboxy end of the protein was used to generate progressively shorter proteins containing BHV-1 gC aa 1–122, aa 1–103, and aa 1–69. Mab F2 recognized all of these proteins, localizing the epitope between aa 1 and 69 (Fig. 5C). Because the mature form of BHV-1 gC probably lacks the signal sequence aa 1–21 (Fitzpatrick *et al.*, 1989), residues 22–69 are likely to contain an epitope recognized by the MAb F2 (Fig. 6). MAb 24 (Fig. 5B) recognized fusion proteins containing gC aa residues 1–122 but did not react to aa residues 1–103 and 1–69, indicating that the location of an epitope is between aa residues 104–122 (Fig. 6).

Similarly, the induced fusion proteins containing BHV-5 gC residues aa 1–300, aa 1–136, and aa 1–78 (represented by p5gC1-300, p5gC1-136, and p5gC1-78, respectively) were recognized by MAbs 8B1 (Figs. 7B and 8) and L6G (data not shown). Because the mature form of BHV-5 gC probably lacks the signal sequence aa 1–30 (Fig. 2), residues 31–78 are likely to contain the epitope recognized by MAbs 8B1 and L6G.

BHV-1- and BHV-5-specific bovine antiserum reacted specifically with fusion proteins containing respective type-specific epitope coding gC sequences

Bovine serum raised against either BHV-1 or BHV-5 whole virus was tested by immunoblotting for reactivity with recombinant fusion proteins containing BHV-1 gC aa residues 22–69 and BHV-5 gC aa residues 31–78. The results (data not shown) showed that anti-BHV-1 and anti-BHV-5 bovine serum reacted specifically with BHV-1 gC aa 22–69 or with BHV-5 gC aa 31–78 containing the respective type-specific antigen. To determine the binding specificity of bovine serum antibodies to fusion proteins, immunoblots containing the BHV-1 or BHV-5 gC residues 22–69 and 31–78 were probed with these bovine sera that were absorbed with purified BHV-1 and/or BHV-5 (Fig. 9). The bovine anti-BHV-5 antibody showed binding specificity to the BHV-5 gC (aa 31–78)-specific

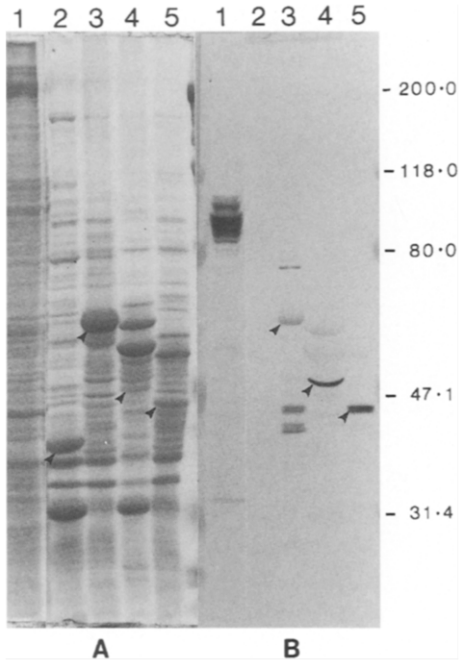

FIG. 7. SDS-PAGE and immunoblotting analysis of trpE-gC (BHV-5) fusion proteins. (A) Coomassie blue-stained gel showing fusion proteins expressed from BHV-5 gC ORF in the pATH vector system. (Lane 1) BHV-5-infected MDBK cell lysate, (lane 2) trpE, (lane 3) trpE-gC (BHV-5) aa 1-300, (lane 4) trpE-gC (BHV-5) 1-136, and (lane 5) trpE-gC (BHV-5) 1-78. (B) Immunoblotting of the same proteins with BHV-5-specific MAb 8B1. The locations of (lane 2) trpE and (lanes 3-5) trpE-gC (BHV-5) fusion proteins are marked with arrowheads. Immunoblotting with MAb L6G (data not shown) revealed identical but weaker reactivity.

fusion protein; its binding was substantially blocked by BHV-5 but not by BHV-1 virus absorption (Fig. 9). Conversely, the bovine anti-BHV-1 antibody showed specificity to BHV-1 gC (aa 22-69)-specific fusion protein, and its reactivity was largely inhibited by BHV-1 but not by BHV-5 virus absorption (Fig. 9). These results indicated that the type-specific epitopes defined by BHV-1- and BHV-5-specific murine MAbs (F2 and 8B1/L6G, respectively) also are recognized by bovine antiserum against BHV-1 and BHV-5 in a type-specific manner.

DISCUSSION

The main purpose of this study was to analyze the molecular basis of antigenic diversity between the gC of BHV-1 and BHV-5. The BHV-5 gC sequence was determined, and the predicted aa sequences of BHV-5 gC were compared with the BHV-1 gC sequences. Second, the location of linear type-specific antigenic determinants was identified in segments of the BHV-1 and BHV-5 gC ORFs by expressing genomic subfragments in *E. coli* and then determining MAb reactivity.

Previous studies have located five linear (conformational-independent) epitopes (I, II, III, VIII, and IX) on three separate domains of BHV-1 gC protein (Fitzpatrick *et al.*, 1990; Marshall *et al.*, 1988). Epitopes I, II, and III were mapped in domain II of gC between residues 22 and 150 (Fitzpatrick *et al.*, 1990). The present results separate the boundaries of two of these three epitopes into discrete segments of domain II. The location of an epitope defined by the neutralizing MAb F2 was mapped within the BHV-1 gC residues 22-69. A prominent hydrophilic peak and several potential antigenic peaks are located within this region. Previously, the location of an epitope specified by the same MAb F2 (epitope IX) was reported to be within residues 140-240 (Fitzpatrick *et al.*, 1990). These results clearly differ from our findings. Based on our data, this type-specific epitope is located at the amino-terminal region of the protein, which showed very little or no homology to the corresponding BHV-5 segment. However, residues 160-505 (BHV-1 gC) showed considerable homology to the corresponding BHV-5 gC segment (aa 124-470). Thus, the BHV-1-specific linear epitope defined by MAb F2 probably would not map in a highly conserved region of the two viruses. Consistent with this supposition is the finding that MAb F2 did not react to the fusion protein containing BHV-1 gC residues 173-336.

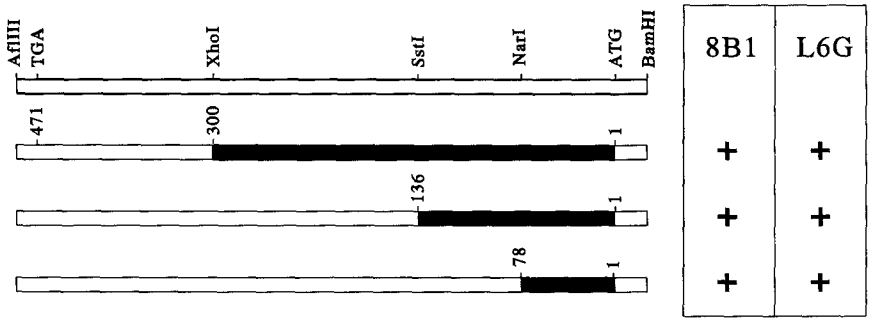

FIG. 8. Schematic representation of the BHV-5 gC glycoprotein and its segments that were expressed in pATH vectors. The *Bam*HI/*Afl*III fragment containing the gC (BHV-5) ORF is depicted on top, showing restriction endonuclease sites used for cloning in pATH plasmids (for details see Material and Methods). Glycoprotein segments expressed are shown as solid bars identified by aa numbers. A summary of the immunoreactivity of gC protein segments with MAbs 8B1 and L6G is shown on the table to the right, with (+) to denote reactivity and (-) for no reactivity.

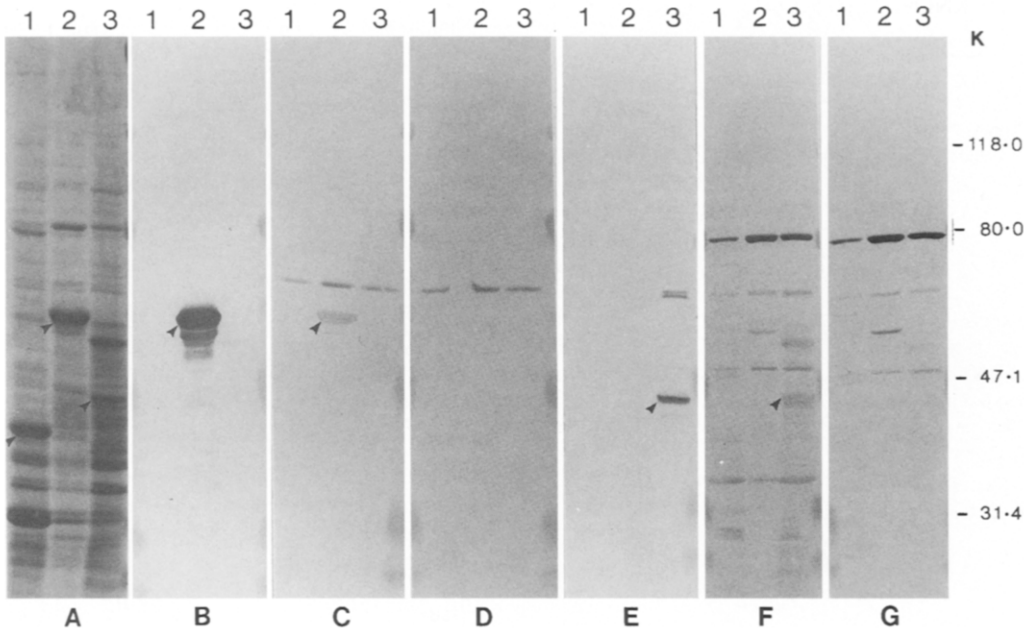

FIG. 9. Type specificity of trpE–gC fusion proteins expressing the N-terminal 1–69 and 1–78 amino acids of BHV-1 and BHV-5 gC, respectively. (A) Coomassie blue-stained SDS–PAGE gel containing (lane 1) trpE, (lane 2) trpE–gC (BHV-1) 1–69, and (lane 3) trpE–gC (BHV-5) 1–78. Immunoblotting of the same proteins with (B) BHV-1 gC 1–69-specific MAb F2, (C) bovine anti-BHV-1 serum absorbed to BHV-5, (D) bovine anti-BHV-1 serum absorbed to BHV-1 and BHV-5, (E) BHV-5 gC 1–78-specific MAb 8B1, (F) bovine anti-BHV-5 serum absorbed to BHV-1, and (G) bovine anti-BHV-5 serum absorbed to BHV-1 and BHV-5. The locations of trpE, trpE–gC (BHV-1) 1–69, and trpE–gC (BHV-5) 1–78 proteins are marked with arrowheads.

BHV-1 gC aa residues 104–122 contained an epitope specified by neutralizing MAb 24. This segment contains one of the most prominent hydrophilic peaks of the BHV-1 gC aa sequence. A potential N-linked glycosylation site (aa residue 111) also is present within this segment. However, MAb 24 reacts very strongly to the virus glycoprotein as well as to *E. coli*-expressed unglycosylated protein. Thus, it is likely that the epitope recognized by MAb 24 is glycosylation independent.

BHV-5-specific MAbs 8B1 and L6G both recognized an epitope coding region containing BHV-5 gC residues 31–78. This region contained two hydrophilic peaks and several potential antigenic peaks. One hydrophilic peak (aa 35–50) was the most prominent in the entire gC coding region. Considering the length of the region, these sequences could specify two distinct epitopes (MAbs 8B1- and L6G-specific).

These results are in agreement with the predicted aa sequence alignment data, because the aa sequences at the amino-terminal segment of BHV-5 and BHV-1 gC are significantly different, and the BHV-1 and BHV-5 gC-specific epitopes defined by the type-specific MAbs also mapped within the amino-terminal segment of the protein. Bovine antisera against BHV-1 and BHV-5 showed specificity to BHV-1 gC residues 22–69 (MAb F2 epitope) and to BHV-5 gC residues 31–78 (MAb 8B1), respectively. The reactivity of MAbs F2 (BHV-1 gC-specific) and 8B1 (BHV-5 gC-specific) also has been consistent with several BHV-1 field isolates and two of three BHV-5 isolates from the United States (S. I. Chowdhury, unpublished data). Thus, the epitope coding sequences defined by MAbs F2 and 8B1 appeared to be type specific. The presence of antibodies to these epitopes may be of diagnostic importance in discriminating between respiratory BHV-1 and neurovirulent BHV-5 infections in cattle.

Several previous reports indicated that BHV-5 gC is slightly smaller than BHV-1 gC (Collins *et al.*, 1993; Friedli and Metzler, 1987). Consistent with these previous findings, it has been shown that the BHV-5 gC ORF is 50 aa shorter in length (471 aa) than the BHV-1 gC ORF (521 aa). The carboxy-terminal two-thirds of the gC is well conserved between the two viruses. This includes the central region of gC (aa residues 172–323) that is reported to be the BHV-1 heparin-binding domain (Okazaki *et al.*, 1994). However, the carboxy-terminal hydrophilic region is considerably shorter in the BHV-5 sequences. Thus, the sequence differences at the amino- and the carboxy-terminal region, the absence of two putative glycosylation sites in the BHV-5, and the differences in the net charge of the amino-terminal regions of BHV-1 and BHV-5 gC could partially contribute to biological properties of these two viruses, including the differential pathogenesis of respiratory and neurological disease. Future studies of BHV-1 and 5 gC should determine if such differences are functionally important in the pathogenesis of respiratory and neurological diseases caused by these two viruses.

ACKNOWLEDGMENTS

The author thanks Drs. J. Collins and G. Letchworth for their suggestions and critical reading of the manuscript, Dr. W. Lawrence for a plasmid clone containing the BHV-1 *Hin*dIII I fragment, and Ray Hiss and Cynthia Foley for technical assistance. This work was supported by USDA Section 1433 formula funds and BRSG funds of KSU to S. Chowdhury. Published as Contribution 95-276-J, Kansas Agricultural Experiment Station.

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